BARD Final Report IS-4592-13

Early post-hatch thermal stress effects on broiler muscle development and performance

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Abstract

In broilers, the immediate post-hatch handling period exposes chicks to cold or hot thermal stress, with potentially harmful consequences to product quantity and quality that could threaten poultry meat marketability as a healthy, low-fat food. This lower performance includes adverse effects on muscle growth and damage to muscle structure (e.g., less protein and more fat deposition). A leading candidate for mediating the effects of thermal stress on muscle growth and development is a unique group of skeletal muscle cells known as adult myoblasts (satellite cells). Satellite cells are multipotential stem cells that can be stimulated to follow other developmental pathways, especially adipogenesis in lieu of muscle formation. They are most active during the first week of age in broilers and have been shown to be sensitive to environmental conditions and nutritional status. The hypothesis of the present study was that immediate post-hatch thermal stress would harm broiler growth and performance. In particular, growth characteristics and gene expression of muscle progenitor cells (i.e., satellite cells) will be affected, leading to increased fat deposition, resulting in long-term changes in muscle structure and a reduction in meat yield. The in vitro studies on cultured satellite cells derived from different muscle, have demonstrated that, anaerobic pectoralis major satellite cells are more predisposed to adipogenic conversion and more sensitive during myogenic proliferation and differentiation than aerobic biceps femoris cells when challenged to both hot and cold thermal stress. These results corroborated the in vivo studies, establishing that chronic heat exposure of broiler chicks at their first two week of life leads to impaired myogenicity of the satellite cells, and increased fat deposition in the muscle. Moreover, chronic exposure of chicks to inaccurate temperature, in particular to heat vs. cold, during their early posthatch periods has long-term effects of BW, absolute muscle growth and muscle morphology and meat quality. The latter is manifested by higher lipid and collagen deposition and may lead to the white striping occurrence. The results of this study emphasize the high sensitivity of muscle progenitor cells in the early posthatch period at a time when they are highly active and therefore the importance of rearing broiler chicks under accurate ambient temperatures. From an agricultural point of view**,** this research clearly demonstrates the immediate and long-term adverse effects on broiler muscling and fat formation due to chronic exposure to hot stress vs. cold temperatures at early age posthatch. These findings will aid in developing management strategies to improve broiler performance in Israel and the USA.

Summary Sheet

Publication Summary

PubType	IS only	Joint	US only
Reviewed	0	3	0

Training Summary

Trainee Type	Last Name	First Name	Institution	Country
M.Sc. Student	Patael	Tomer	Hebrew Univesrsity	Israel
Ph.D. Student	Hardaman	Rachel	Ohio State University	USA

Collaborations:

Collaborations between the groups in IS and the USA were on steady basis:

1. All the RNA samples derived from the experimental chicks in the in vivo studies were sent from IS to USA for gene analysis.

2. Two successful visits to IS on the second year and to USA on the third year were conducted by Sandra Velleman and Orna Halevy, respectively. In each visit, the visiting collaborator gave departmental or institutional seminars regarding the BARD results and these were followed by discussions both, between the collaborators and with other departmental members.

3. Data sharing and discussions were performed on a regular basis during the entire project period.

4. Three papers have already been published as true collaborations and the fourth is under preparation.

Achievements

During the course of this study, we demonstrate that chronic exposure of chicks to inaccurate temperature, in particular to heat vs. cold, during their early posthatch periods has long-term effects of BW, absolute muscle growth and moreover, on muscle morphology and meat quality. The latter is manifested by higher lipid and collagen deposition and may lead to the white striping occurrence. The main reason for these adverse effects is due to the fact that the chronic exposure of chicks to incorrect temperatures takes place at periods crucial for muscle development when the muscle progenitor cells (i.e., satellite cells) are most active. Indeed, our in vitro studies clearly demonstrate that satellite cells are very sensitive to temperature changes in respect to proliferation and differentiation (Harding et al., 2016) and change their fate towards the adipogenic lineage (Harding et al, 2015). The in vivo studies corroborate the in vitro ones (Piestun et al., 2017). Our previous reports in which short and specific heat manipulations had promotive effects on muscle development, together with these data, imply a specific effect of heat-stress level and duration at critical periods of SC activity during early growth phase of broilers which could lead to opposite, reducing vs. improving effects on muscle growth.

From an agricultural point of view**,** this research clearly demonstrates the immediate and long-term adverse effects on broiler muscling and fat formation due to chronic exposure to hot stress vs. cold temperatures at early age posthatch. These findings will aid in developing management strategies to improve broiler performance in Israel and the USA.

Changes to the original plan

The original objects were as follow:

1. Objective 1 (USA) to determine the effect of temperature on SC proliferation, differentiation, transdifferentiation to adipocytes, and apoptosis.

2. Objective 2 (Israel and USA) to determine the effect of various immediate post-hatch rearing temperatures (i.e., TS) on SC proliferation and differentiation, muscle development, fat deposition and apoptosis in post-hatch broilers.

3. **Objective 3 (Israel and USA)** to determine the effect of various immediate post-hatch TS on broiler performance to market age.

4. Objective 4 (Israel and USA) to determine the optimal TM and the long-term effects of the optimal TM during embryogenesis in mitigating immediate post-hatch TS effects on SC proliferation and differentiation, transformation to adipocytes, apoptosis, and broiler performance to market age.

While Objectives 1-3 had been fulfilled and most the results were published or in preparation for publication, Objective 4 had not been accomplished due to technical and personnel problems, which caused delays in the conduction of Objective 2 and 3. Yet, Objective 3 had been elaborated and more analyses were included such as, collagen deposition in muscle and specific immunostainings for adipogenic proteins (see appendix).

Publications for Project IS-4592-13

Status	Type	Authors	Title	Journal	Vol:pg Year	Coun
Published	Reviewed	*Harding, R.L., Clark, D.L., Halevy, O., Coy, C.S., Yahav, S., and Velleman, S.G.*	The effect of temperature on apoptosis and adipogenesis on skeletal muscle satellite cells derived from different muscle types	***Physiological Reports***	3 : e12539 2015	Joint
Published	Reviewed	*Harding, R.L., Halevy, O., Yahav, S., and Velleman, S.G*	The effect of temperature on proliferation and differentiation of skeletal muscle satellite cells isolated from different muscle types	***Physiological Reports***	4 : e12539 2016	Joint
Published	Reviewed	*Piestun, Y., Patael, T., Yahav, S., Velleman, S.D., Harding, R.L. Coy, C.S., and Halevy, O.*	Early post-hatch thermal stress affects broiler muscle development and satellite cell fate	***Poultry Science***	: 2017	Joint

Appendix 1:

Early Post-Hatch Thermal Stress Effects on Broiler Muscle Development and Performance- IS-4592-13

Final Report

Introduction

The physiological response of chicks to thermal stress (TS) may initiate an irreversible cascade of events that in extreme cases can be lethal. Thermal stress may also lead to muscle damage with associated changes in product quality (Gregory 1994, 1998; Mitchell, 1999). Chicks experience TS around the time of hatch. Although the hatching of chicks is synchronized, it can still last up to 36 h, a period in which the new hatchlings are kept in the incubator without access to food or water and exposed to high hatchery temperatures of approximately 37.5°C. In addition, the immediate post-hatch handling and shipping period can last up to 2 days, and during this period chicks can be exposed to variable temperatures including both heat and cold stresses, which can harm the birds' well-being and physiological responses (Mitchell and Kettlewell, 2009). All the aforementioned factors will result in economic losses for producers and processors. Studies on broilers have demonstrated that chronic heat exposure results in lower weight gain, coupled with a lower ratio of breast muscle weight to body weight (BW), and increased intermuscular fat deposition (Baziz et al., 1996; Yahav et al., 1996). Physiological and molecular responses to TS (cold and hot) must be understood to ensure quality poultry products. Moreover, management strategies concerning TS are needed to maximize muscle growth and to reduce developmental changes affecting meat quality.

A leading candidate for mediating the effects of TS on muscle growth and development in postnatal growing vertebrates is the satellite cells (SC), which are located between the basement membrane and sarcolemma of the myofibers. These cells, also termed adult myoblasts, are most active in the late-term embryo and post birth/hatch. In broilers, SC are mainly active in the first week of posthatch age, after which their proliferative activity rapidly declines and they become mainly quiescent. Thus, management efficiencies, especially ambient temperature (T_a) during the period of maximal SC activity immediately post-hatch, will likely impact long-term performance including both the muscle fibrillar structure and muscle weight. Moreover, SC are now categorized as multipotential stem cells. In vitro studies have shown that SC can be induced to follow myogenic, osteogenic, or adipogenic cellular pathways (Asakura et al., 2001, Shefer et al., 2004; Vettor et al., 2009). As such, SC can be induced to follow other cellular lineages when they are most active immediately after hatch. Therefore, SC may respond

differentially to various thermal conditions with varied impacts on muscle development and growth**.** Previous studies have shown that in broilers, increasing incubation temperature at critical time-points for muscle development or short mild heat stress in the early post-hatch period causes accelerated proliferation and differentiation of SC and enhances muscle growth at later ages (Halevy et al., 2001; Piestun et al., 2009a,b, 2011, 2015). In contrast, our preliminary studies had shown that a more severe heat or cold stress in broilers during the first two weeks of age had an opposite effect on these parameters. Thus, the hypothesis for the proposed research was that immediate post-hatch TS will alter broiler performance including SC growth characteristics and gene expression, leading to increased fat deposition, and will result in long-term changes in muscle structure and a reduction in meat yield.

During the first and second year the in vitro results revealed that incubation of SC under different temperatures affects their proliferation, differentiation and apoptosis processes, and that SC derived from the pectoralis muscle are more predisposed to adipogenic conversion than the ones derived from the femoris when thermally challenged **(Objective 1**). The results emanating from this study were published in two papers (Harding et al., 2015, 2016). An in vivo experiment in which chicks were reared under two temperature schemes for the first two weeks posthatch (**Control** vs. **Hot**, see Table 1), demonstrated a reduction in the body and breast muscle growth as well as a reduction the number of SC as a result of increasing the breeding temperature from day 1 of age onward until day 13 of age (**Objective 2**). Moreover, in agreement with the result from **Objective 1**, lipid deposition was observed in SC derived from the heat-treated chicks on days 8 and 13 of age and in pectoralis muscle cross sections from day 13, whereas the controls barely exhibited any lipid staining. The lipid deposition accompanied an increase in the gene expression of adipogenic genes. These results were recently published (Piestun et al., 2017). Together, the results emerging from **Objectives 1 and 2** suggested high sensitivity of muscle progenitor cells in the early posthatch period at a time when they are highly active, to chronic heat exposure, leading to impaired myogenicity of the satellite cells and shifting cellular fate to an adipogenic lineage. The next goal of this study was to explore the long-term effects of TS of various temperatures during posthatch period on muscle development, growth, and chicks' performance (**Objective 3**).

Methods

According to **Objective 3**, a long-term in vivo experiment was conducted under four different temperature regimes shown in Table 1. On various days until day 35, chicks (n=10) were weighted and body temperature was determined. Pectoralis muscle was sampled from

parallel chicks (n= 7) for protein and mRNA analyses and for histology assays. For RNA analysis, samples were quickly put in an RNAsafe solution and kept in -20 °C until their shipping to USA for gene analysis. For morphological and immuno-histological assays [e.g., hematoxylin and eosin (H&E), proliferating cell nuclear antigen (PCNA), TUNEL], samples were embedded in paraffin. For lipid analysis in muscle (Oil Red O staining), pectoralis muscle was sampled on various days, snapped-frozen in liquid nitrogen, and kept at -80^{o}C for further analysis.

Results

In the long-term experiment (**Objective 3**), the effect of exposure to hot, mild-hot or cold temperatures for 13 days was examined on growth parameters until day 35. Body temperature was monitored until day 27 and was the highest in the Cold group and the lowest in the Hot and Hot Mild groups (Fig. 1). Body weight (BW) remained the lowest throughout the entire experiment in the Hot group, while that of the Cold group remained similar to that of the Control (Fig. 2). The BW of the Hot Mild group was lower than that of Control on days 6 and 21, reaching back the Control group BW levels on later days. Similar pattern was observed with respect to the breast muscle (pectoralis major and minor) weight (Fig. 3). Interestingly, the Cold group had the lowest breast muscle weight only on day 3, probably because the chicks deposited the main energy for survival rather than to growth. Absolute breast muscle growth (% of BW) was the lowest in the Hot group from day 8 onward (Fig. 4), while the percentage of abdominal fat of BW was higher in the Hot and Hot Mild groups compared to these in Control and Cold groups on day 35 (Fig. 5).

The lower absolute muscle growth in the Hot group could be explained by a lower rate of hypertrophy relative to the other groups. Indeed, the average myofiber diameter on day 8, 13 and 35 of age was lowest in the Hot group (Table 2); diameter distribution curves showed that on all days the Hot-group curve was the most shifted to the left, to the smaller diameter bins (Fig. 6, see also Fig. 8). On the other hand, and in agreement with the relatively highest breast muscle percentage (Fig. 4), the Cold group presented the highest myofiber diameter average (Table 2).

The lower hypertrophy of the Hot group on day 35 was accompanied with higher fat deposition in the interstitial areas in the pectoralis major muscle (Fig. 7). A morphology analysis by H&E staining of the pectoralis muscle sections revealed large white areas in between the myofiber areas, resembling fat droplets (Bailey et al., 2015) in the Hot and to a lesser extent in the Hot Mild muscle sections (Fig. 7). Fat deposition was observed also in the Control group;

however, the fat droplets were much smaller than those in the Hot and Hot Mild muscle sections. An Oil Red staining of muscle cryo-sections revealed similar results (Fig. 8).

Our previous and current results suggested that the inappropriate temperature which the chicks were exposed to in the first two weeks of age leads to an increase in muscle fat deposition and even to a change in the SC fate to the adipogenic lineage (Harding et al., 2015; Piestun et al., 2017). One of the key proteins in the adipogenic lineage is C/EBPβ which have been reported to be induced during the trans-differentiation of myocytes into adipocytes (Yu et al., 2006), and is often used as a marker for adipogenesis. A gene analysis assay for C/EBPβ and peroxisome proliferator-activated receptor gamma (PPARγ) in pectoralis muscle samples derived from chicks on day 6 posthatch did not reveal any significant difference between the treatments (Table 3). Unfortunately and as opposed to our previous studies (Piestun et al., 2017), there were no statistical difference in the C/EBPβ mRNA levels in the Control vs. Hot groups on day 8 (Table 3). The C/EBPβ mRNA levels of the Cold and unexpectedly of the Hot Mild groups were lower than that of the Control. In light of these results, an additional immunoassay was conducted to analyze the protein expression and location of C/EBPβ in the pectoralis muscle sections from 8-day-old chicks. Fig. 9A depicts the staining of myonuclei underneath the basal membrane of the myofiber and in a central nucleus, suggesting a positive staining of SC nuclei. Quantitation of the number of C/EBPβ–positive nuclei out of total nuclei showed a marked increase in the number of these nuclei in the Hot group and to a lesser extent in the Hot Mild groups, relative to the Control and Cold groups; the latter groups presented similar values (Fig. 9B).

The increase in fat deposition in the Hot and Hot Mild pectoralis muscle (Fig. 7 and 8) raised the possibility of increase in collagen content in the muscle that is associated with myo-degeneration (Bauermeister et al., 2009). A Sirius Red staining for collagen deposition in the pectoralis muscle sections revealed more collagen-positive areas in the Hot and Hot Mild groups compared to the other groups (Fig. 10A). Collagen was deposited in large bundles in most of the interstitial areas in the Hot and Hot Mild muscle sections (note the fat droplets marked with arrows in the Hot muscle section). In contrast, there were hardly collagen bundles in the Control and Cold muscle sections. Indeed, a quantitation analysis for collagen content demonstrated a significant higher collagen content in the Hot and Hot Mild groups and the lowest collagen content in the Cold group compared to that in Control (Fig. 10B).

In order to address the early events that led to the impaired muscle hypertrophy and morphology we addressed the proliferation and survival of SC as well as adipogenic gene and

protein expression in pectoralis muscle sections derived from the experimental chicks on day 8. Proliferation of SC in muscle was evaluated by immunostaining for the cell-cycle marker protein, PCNA (Fig. 11). The number of PCNA-positive nuclei (i.e., SC) out of total nuclei was the highest in the Cold group and the lowest in the Hot and Hot Mild groups. In contrast, the number of TUNEL-positive nuclei out of total nuclei was similar among the groups and ranged between 2-3%, suggesting that the various temperature had not affected SC survival (Fig. 12), in agreement with our previous results (Harding et al., 2015; Piestun et al., 2017)

Discussion

The physiological parameters of chicks found in the long-term, experiment are in agreement with those of the second experiment (see second year report). Along the entire experiment until day 35, the severest worsening effects with respect to BW, pectoralis weight and abdominal fat were observed in the chicks that were exposed to chronic high temperatures in the first two weeks posthatch (Hot group). Exposure to milder heat (Hot Mild) also worsened these parameters relative to the Control, while exposing chicks to cold temperatures (Cold) had no effect at least from day 6 onward. The absolute muscle growth as determined by % breast muscle weight of BW was the lowest in the Hot group on all days, reflecting a lower hypertrophy of the pectoralis muscle fibers due to the severe TS. Indeed, the morphology of pectoralis muscle sections depicted smaller myofibers in the Hot group (Fig. 7). In addition, the diameter average of these myofibers was the lowest in this group on day 35 as well as the distribution of the myofiber diameter; the curve was shifted to the left, to the lower diameter bins (Fig. 6, Table 2). The impaired hypertrophy was probably due to a direct effect of the heat stress, as the curve shift was observed already on days 8 and 13 posthatch. Moreover, a significant reduction in the SC proliferation was demonstrated in the pectoralis muscle of the Hot group on day 8 (Fig. 11) , suggesting a reduction in the progenitor cell reservoir which is crucial for future hypertrophy (Halevy et al., 2000, 2006; Piestun et al., 2009). Interestingly, while the Hot Mild group presented similar results to the Hot group with respect to myofiber hypertrophy, PCNA levels on early age posthatch along the experiment, by day 35 absolute muscle growth was regained and hypertrophy returned back to those of the Control group. These results suggest that the mild-heat stress caused a temporary damage in muscle growth, which was recovered later on, whereas the damage caused by the high heat stress was permanent.

The cold stress, caused a significant lower muscle weight and absolute muscle growth relative to control temperatures only in the first days posthatch (Fig. 3 and 4). These results are

in agreement to those observed in the in vitro experiments where SC were exposed to various temperatures from 33^{o}C to 42^{o}C for 72 h; the proliferation was the lowest when SC were exposed to 33^{o}C (Harding et al., 2016)**.** However, in contrast to the long-term effects caused by the heat stresses either severe or mild, the reduction in muscle weight by the cold stress was transient as the weight levels recovered back to the levels demonstrated in the Control group as early as on day 6 posthatch. Indeed, the PCNA levels of SC in this group were the highest on day 8, implying that under chronic cold conditions, chicks can recover from the initial cold stress on the first days posthatch and later on compensate for the impairment in muscle growth. From the applicative point of view, an exposure to colder temperature during the first two weeks of age could be could be an approach to boost SC proliferation to enlarge its reservoir for long-term muscle growth.

Another aspect related to the broiler's body and muscle growth—fat deposition—was analyzed in this project. Increases in abdominal fat deposition was observed in the Hot and Hot Mild groups compared to Control and Cold groups on day 35 (Fig. 2). Moreover, higher fat deposition was observed in these groups in the pectoralis muscle. In fact, the increase in fat deposition in myoblasts and myofibers that was found on day 8 and 13 in the Hot group (**Objective 2**; Piestun et al., 2017) was now manifested in myofibers in this and Hot Mild groups on day 35 as demonstrated by Oil Red staining (Fig. 8) and also by the large lipid droplets found in the interstitial areas between the myofibers (Fig. 7). Intramuscular fat deposition in broilers occurs under various stress conditions such as food restriction (Velleman et al., 2010, 2014), delayed feeding (Powell et al., 2016) and TS (Baziz et al., 1996; Yahav et al., 1996).

The gene analysis (by RT-qPCR) of adipogenic regulatory factors, C/EBPβ and PPARγ, in pectoralis muscle on day 6 and 8 of pectoralis muscle of the second experiment showed no major differences between the groups (Table 3). It could indeed be that unlike the physiological parameters, the hot-treated groups do not present major difference from the control with respect to alterations in the adipogenic lineage. However, this explanation is less likely because an immunostaining for C/EBPβ of muscle sections from day 8 clearly showed C/EBPβ –positive nuclei in the SC position in the myofibers of the Hot chicks, and higher number of these nuclei in the Hot and Hot Mild groups compared to Control and Cold groups (Fig. 9). Together with the higher fat deposition observed in former groups on day 35 the data supports a transition to the adipogenic lineage in SC and myofibers. The more likely explanation for the lack of significance in the gene analysis results could be due to a technical problem in sampling (e.g., incorrect sampling day).

Taken together, the results imply that continuous heat-stress-induced fat deposition in SC and later on in myofibers could be due to the fusion of SC transdifferentiated to an adipogenic lineage, into myofibers. Satellite cells are considered to be adult muscle stem cells with the ability to differentiate under certain conditions into other cell lineages, including adipogenic and osteogenic cell types (Asakura et al., 2001; Shefer et al., 2004).

Our results revealed a significant increase in collagen content in the Hot and Hot Mild pectoralis muscle vs. the Control and Cold groups on day 35 (Fig. 10). Indeed, in many cases accumulated lipids in skeletal muscles is accompanied with an accumulation of collagen, both are typical features of myopathies. Recently, a phenomenon called the "white striping" has been observed in breast muscle especially in the fast-growing broilers (e.g., Cobb strains) (Bauermeister et al., 2009). White striping is characterized by the appearance of stripes parallel to the myofibers, especially in breast muscle fillets, but it can also be found in thighs. The condition is characterized by localized lipidosis associated with necrotic myofibers and developed connective tissues (i.e., fibrosis). The myo-degenerative myofibers are mainly those showing the highest mean cross-sectional area, while samples with severe myo-degeneration have myofibers of different diameters and without the characteristic polygonal shape. It is believed that a lower capillary-to-myofiber ratio, and greater interpapillary distance is related to the myo-degeneration. The lack of vascularization could be due to genetic selections for heavy breast muscle, excess T3 levels, or suboptimal husbandry conditions one of which could be inaccurate temperature of rearing. The results of this study support the notion that chronic exposure of chicks to mild or high temperatures for the first two-week post-hatch play a role in the striping appearance on later ages. In contrast, exposure to colder temperature may reduce the striping process and myopathy by lowering the collagen and lipid deposition in the muscle. It is plausible that the colder temperature that initially reduces muscle growth (Fig. 2,3), later on encourages vascularization in the muscle, thus preventing its degeneration. A similar response has been recently shown in a genetic line for featherless chicks; they suffer from cold stress in their early days post hatch but later on response in enhanced vascularization and increase in absolute muscle growth (Hadad et al., 2014a, b).

In conclusion, during the course of this study, we demonstrate that chronic exposure of chicks to inaccurate temperature, in particular to heat vs. cold, during their early posthatch periods has long-term effects of BW, absolute muscle growth and moreover, on muscle morphology and meat quality. The latter is manifested by higher lipid and collagen deposition and may lead to the white striping occurrence. The main reason for these adverse effects is due

to the fact that the chronic exposure of chicks to incorrect temperatures takes place at periods crucial for muscle development when SC are most active. Indeed, our in vitro studies (**Objective 1**) clearly demonstrate that SC are very sensitive to temperature changes in respect to proliferation and differentiation (Harding et al., 2016) and change their fate towards the adipogenic lineage (Harding et al, 2015). The in vivo studies (**Objective 2 and 3**) corroborate the in vitro ones (Piestun et al., 2017). Our previous reports in which short and specific heat manipulations had promotive effects on muscle development (Halevy et al., 2001; Piestun et al., 2009, 2013), together with these data imply a specific effect of heat-stress level and duration at critical periods of SC activity during early growth phase of broilers which could lead to opposite, reducing vs. improving effects on muscle growth.

From an agricultural point of view**,** this research clearly demonstrates the immediate and long-term adverse effects on broiler muscling and fat formation due to chronic exposure to hot stress vs. cold temperatures at early age posthatch. These findings will aid in developing management strategies to improve broiler performance in Israel and the USA.

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Figures:

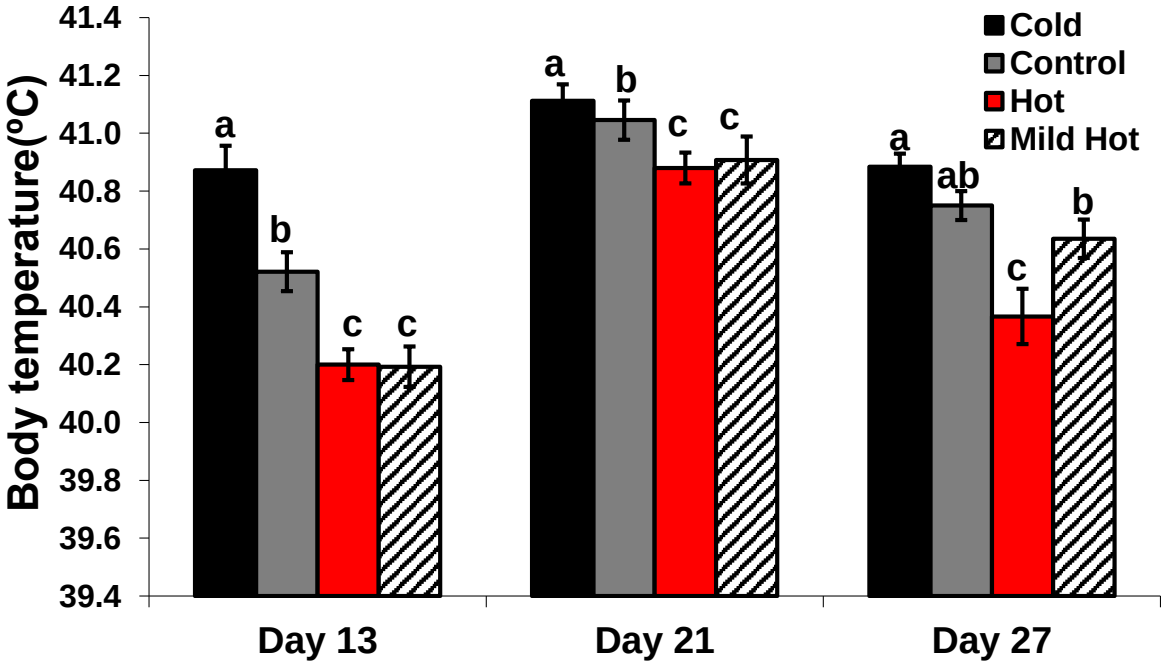

Figure 1: Body temperature at various days of age (n=40). Data with different letters indicates significant difference between groups at the same day ($P \leq 0.05$).

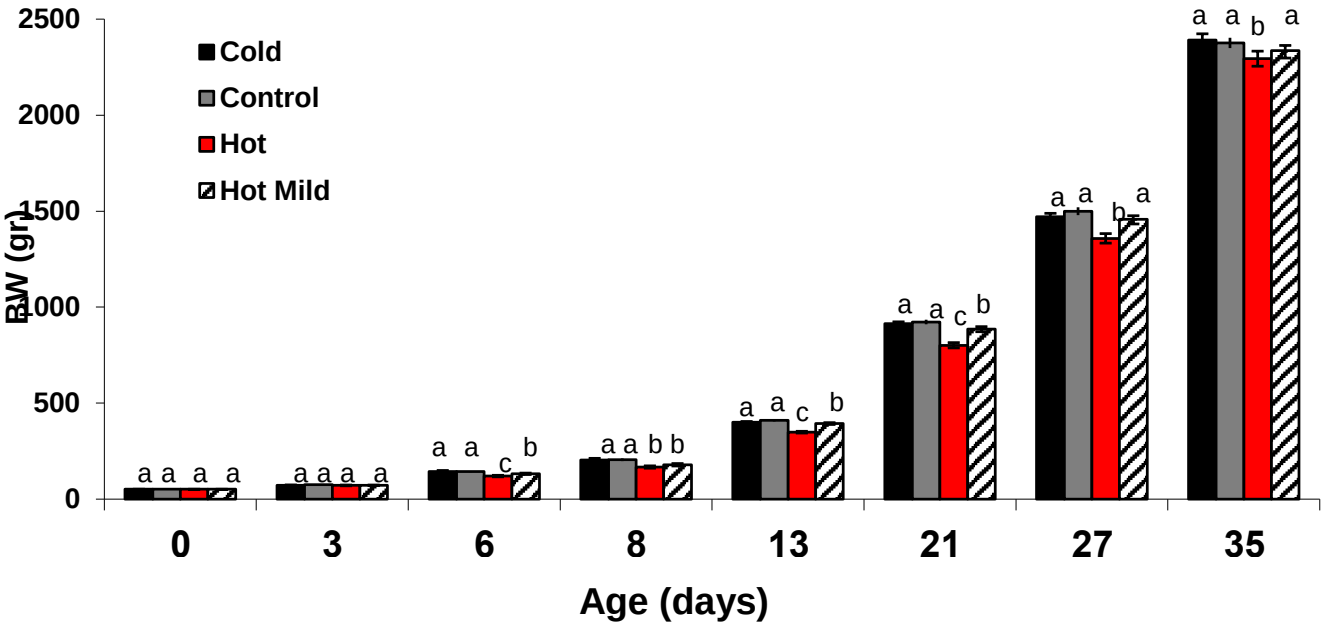

Figure 2: Body weight of the experimental chicks (n = 40). Data with different letters indicates significant difference between groups at the same day ($P \leq 0.05$).

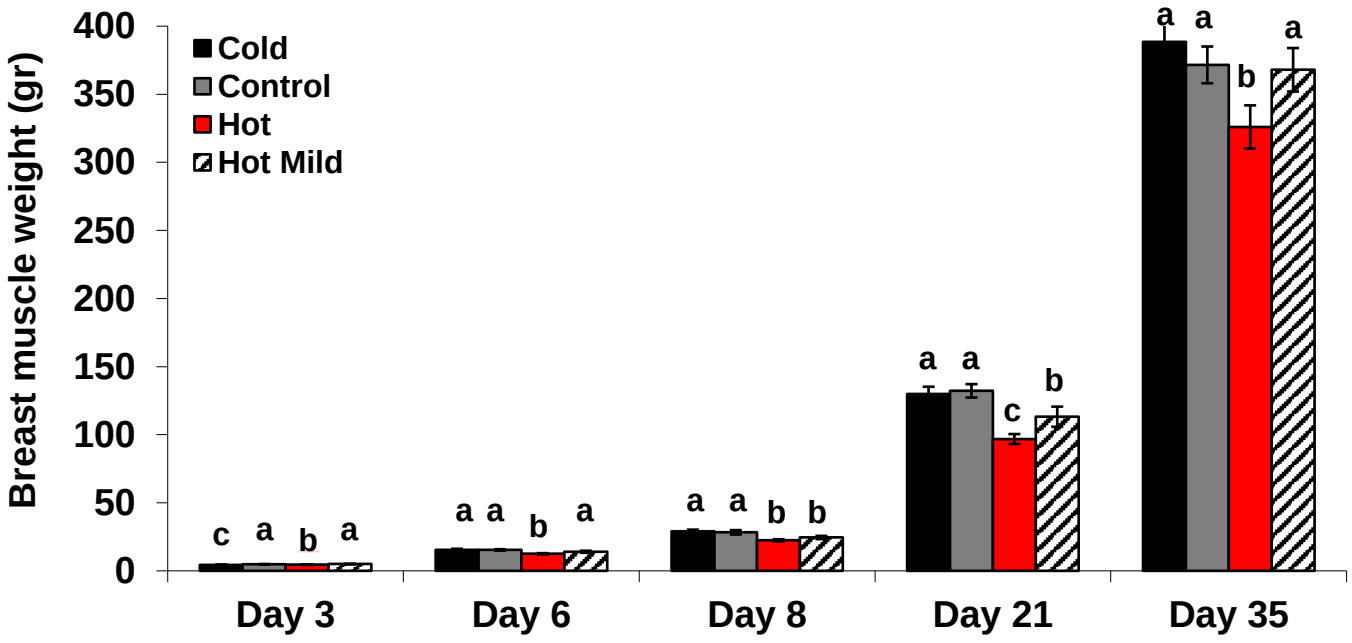

Figure 3: Breast muscle weight (pectoralis major + minor + sternum) of the experimental chicks (n = 10). Data with different letters indicates significant difference between groups at the same day ($P \leq 0.05$).

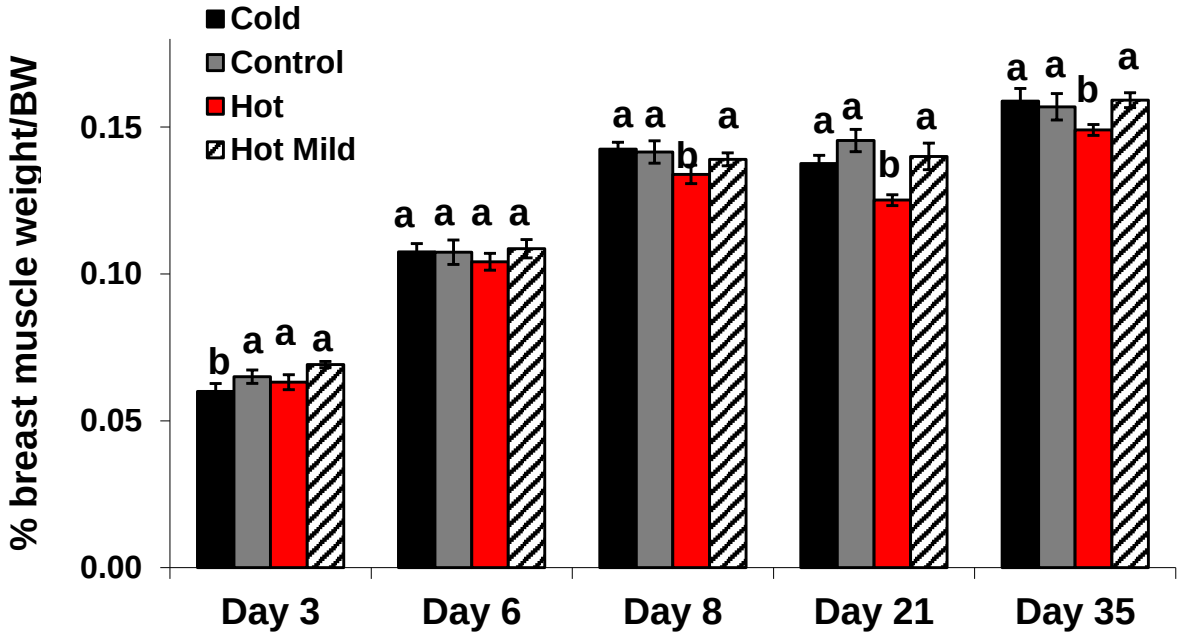

Figure 4: Percentage of breast muscle weight of BW the experimental chicks (n = 10). Data with different letters indicates significant difference between groups at the same day ($P \leq 0.05$).

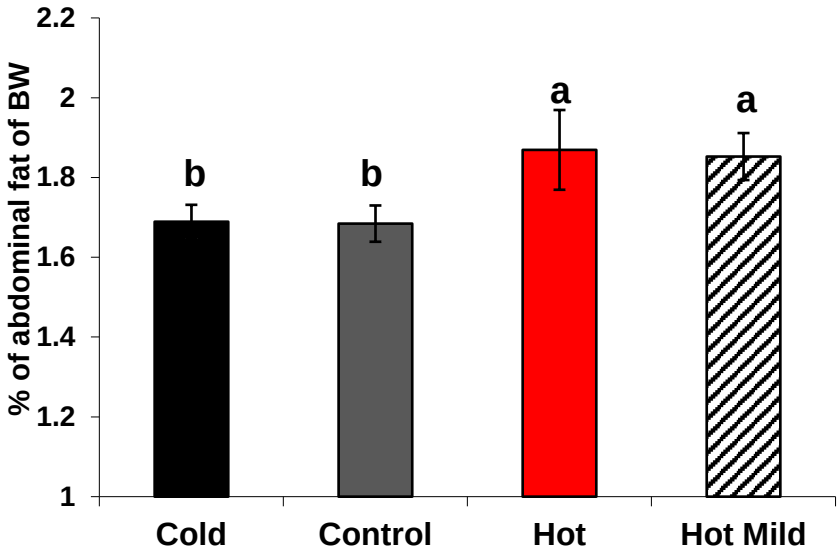

Figure 5: Percentage of abdominal weight of BW the experimental chicks on day 35 (n = 10). Data with different letters indicates significant difference between groups ($P \leq 0.05$).

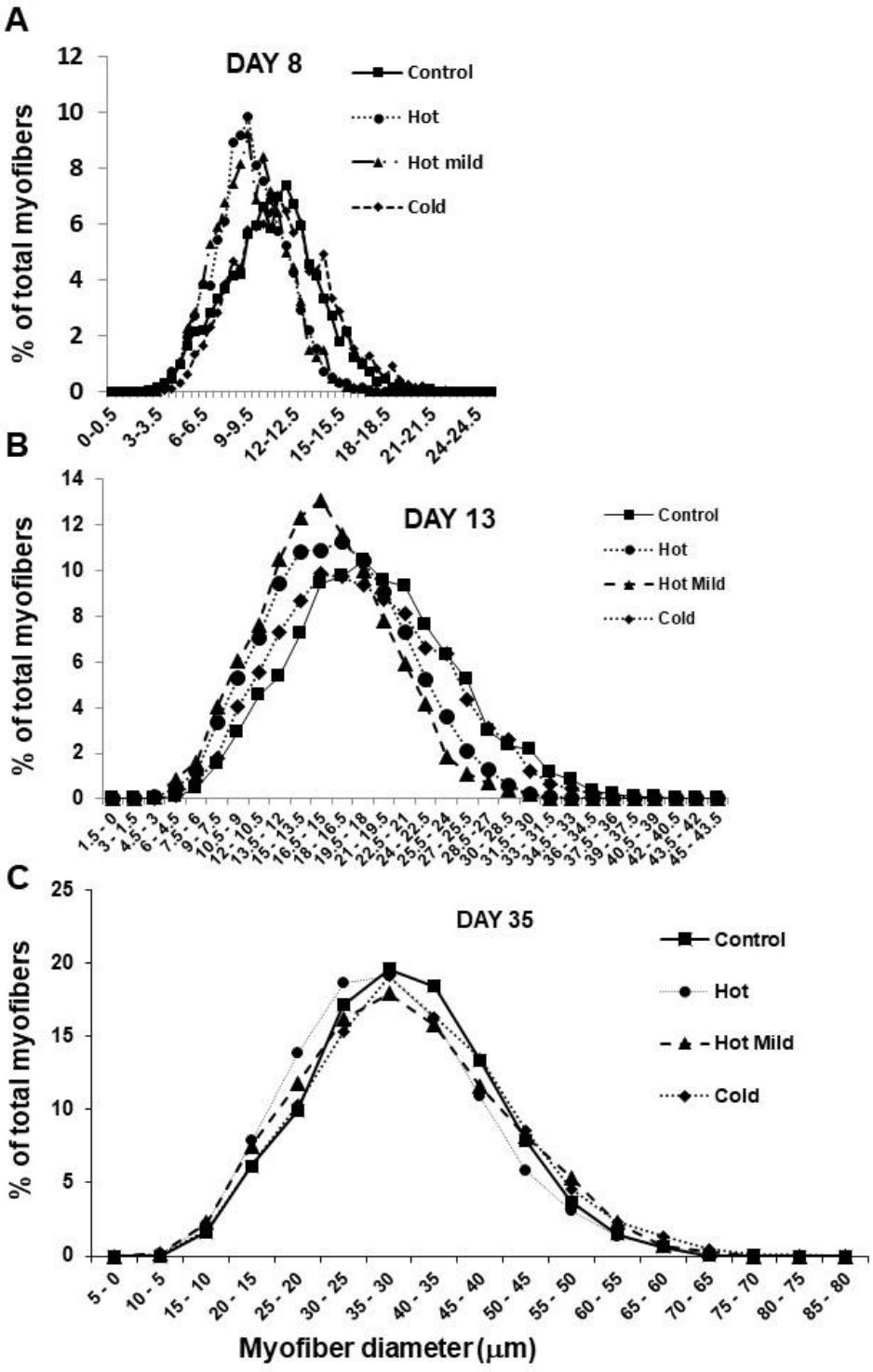

Fig. 6: Myofiber diameter distribution in pectoralis muscle from the experimental chicks on day 8 (A) 13 (B) and 35 (C). Between 4,300 and 4,500 myofibers were counted for each group on each day.

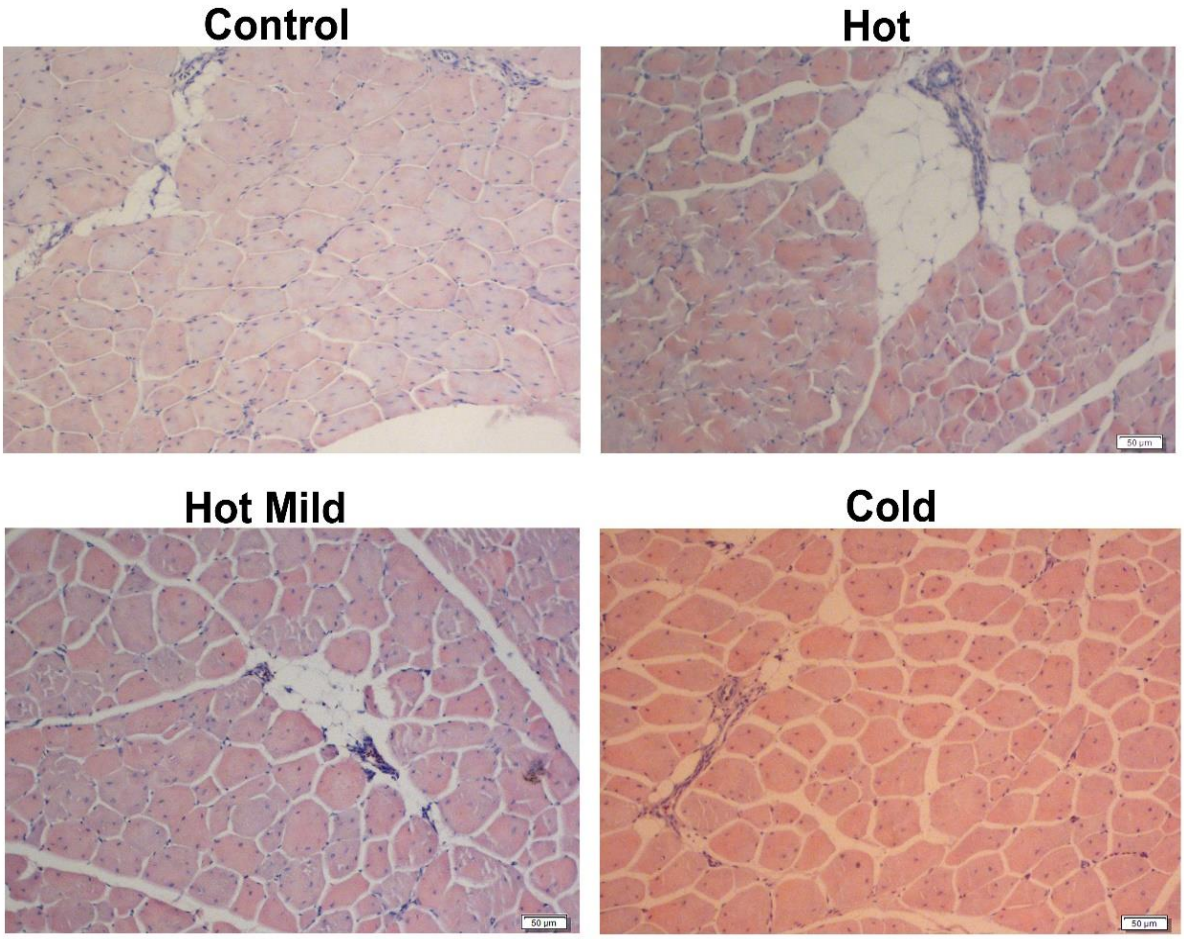

Fig. 7: The morphology of pectoralis muscle derived from the experimental chicks at day 35 of age and stained for H&E. Note the relatively smaller myofiber diameters in the Hot groups as compared with the other groups. Also, the large areas of fat deposition in the Hot and the Hot Mild groups.

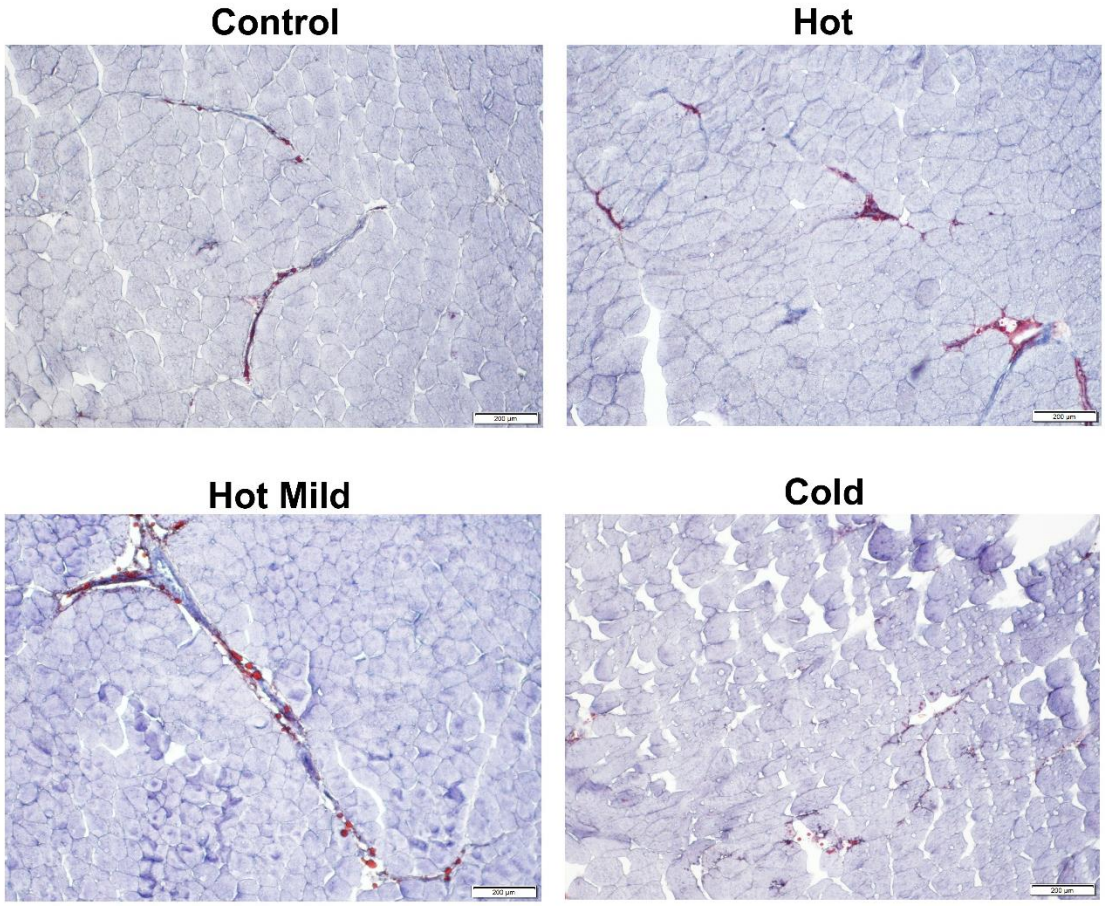

Fig 8: Oil red Staining

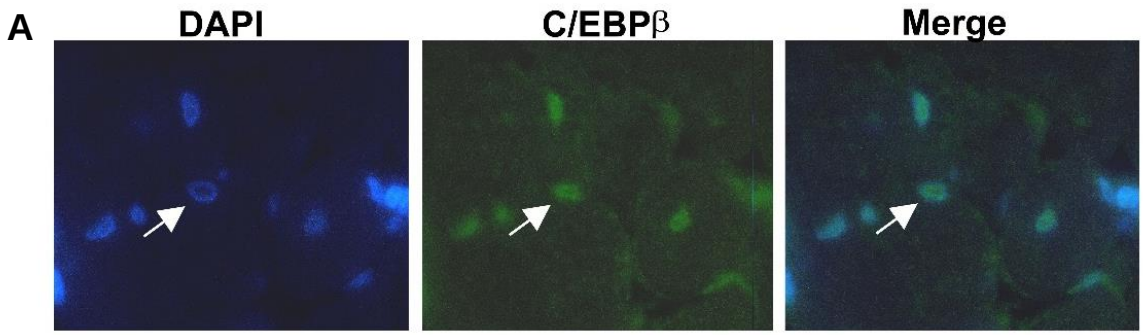

Fig. 9: C/EBPβ expression in pectoralis muscle in the hot-treated chicks on day 8 of age (A). (B) Quantitative analysis of C/EBPβ-positive nuclei presented as percentage of total DAPI-stained nuclei. Values are expressed as means ± SE of three independent chicks in which more than 400 cells were examined. Only nuclei within the myofiber perimeter were included in this analysis. Data with different letters indicates significant difference between groups ($P \leq 0.05$).

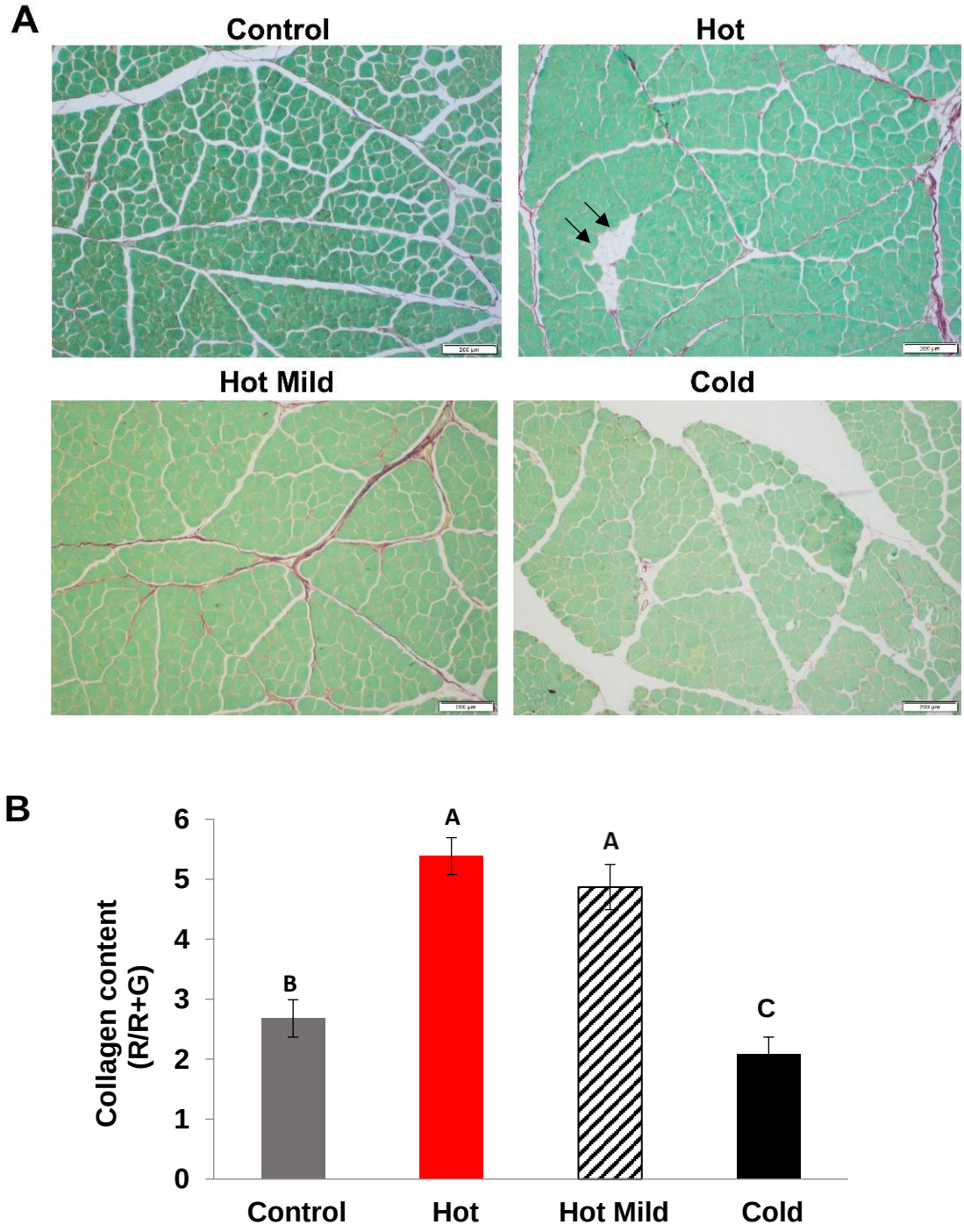

Fig. 10: Effect of temperature exposure for the first two weeks posthatch on collagen synthesis in the experimental chicks. (A) Sirius Red staining of pectoralis muscle sections from day 35 of age. Collagen is stained red (R). Note the high level of collagen in the Hot and Hot Mild groups compared with that in the Control and Cold groups. Also, the large areas of fat deposition (droplets) between inside the myofiber bundles (arrows). (B) Statistical analysis of collagen content in the pectoralis muscle of the experimental chicks. Columns with different letters within each age group differ significantly ($P < 0.05$).

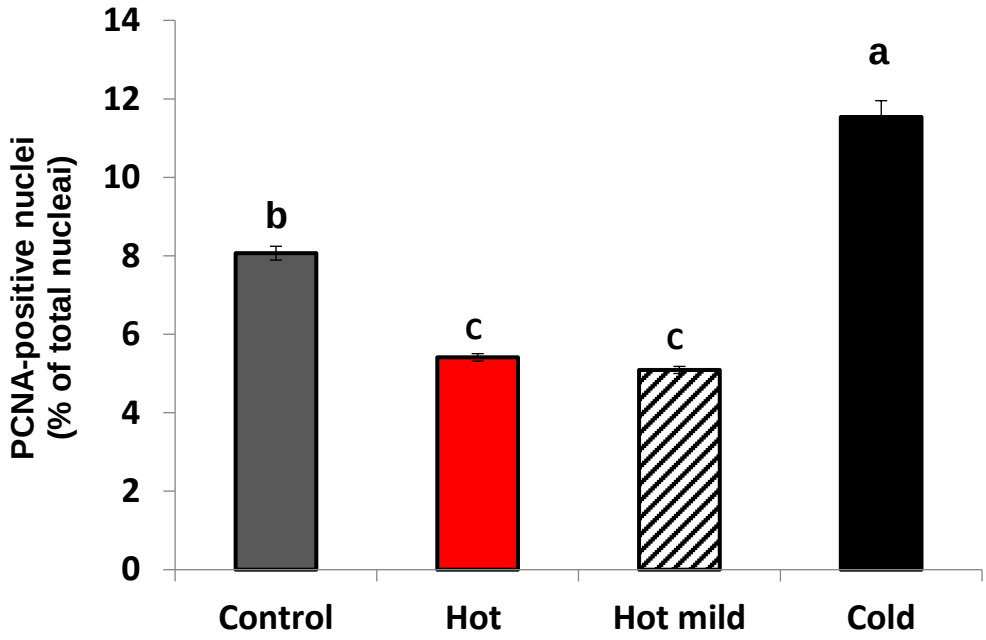

Fig 11: Quantitation analysis of the PCNA-expressing cells. Results are means ± SE of PCNA-expressing cells within the myofibers and are presented as percent of total myonuclei. Five sections were studied per three chicks (n = 3), monitoring seven random fields per section (total of 1500 nuclei per chick) (P < 0.05). Only nuclei within the myofiber perimeter were included in this analysis and regions rich with connective tissue were not included.

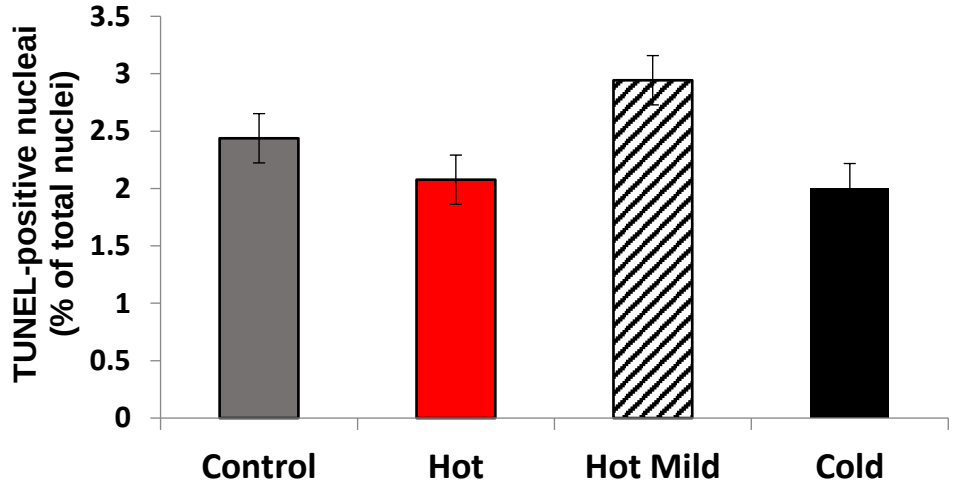

Fig. 12: Quantitative analysis of TUNEL-positive nuclei presented as percentage of total DAPI-stained nuclei. Values are expressed as means ± SE of three independent chicks in which more than 400 cells were examined. Only nuclei within the myofiber perimeter were included in this analysis.

Table 1: Temperature regime in the temperature rooms.

	Cold (°C)	Control (°C)	Hot (°C)	Mild Hot (°C)
Day 0-4	29	**33**	39	35
Day 4-6	28	**32**	37	34
Day 6-8	28	**30**	34	32
Day 8-11	27	**29**	33	31
Day 12-13	27	**28**	33	30
Day 14-25	25	25	25	25
Day 26-35	23	23	23	23

Table 2: Myofiber diameter average of the pectoralis muscle of the experimental chicks on various days of the experiment.

	Cold	Control	Hot	Hot Mild
Day 8	11.44 ± 0.47^{a}	10.90 ± 0.32^{a}	9.41 ± 0.13^{b}	9.37 ± 0.19^{b}
Day 13	18.69 ± 0.65^{ab}	19.74 ± 0.97^{a}	13.88 ± 0.41^{b}	15.65 ± 0.55^{b}
Day 35	34.95 ± 0.17^{a}	34.31 ± 0.15^{b}	32.47 ± 0.15^{c}	34.00 ± 0.17^{b}

Values are expressed as means ± SE of 3 independent chicks in which more than 4,000 myofibers examined per each chick muscle. Data with different letters indicates significant difference between groups ($P \leq 0.05$).

Table 3: Adipogenic regulatory factor gene expression in pectoralis major muscle

C/EBPβ				
Treatment / Day	**Cold**	**Control**	**Hot**	**Hot Mild**
Day 6	5.93± 0.82	8.17±1.42	7.64± 1.04	7.09± 0.87
Day 8	6.47±0.47^{b}	9.63±0.97^{a}	8.38±0.76^{ab}	5.73±0.91^{b}
PPARγ				
Treatment / Day	**Cold**	**Control**	**Hot**	**Hot Mild**
Day 6	0.49± 0.06	0.42±0.08	0.43± 0.05	0.35± 0.02
Day 8	0.34±0.05	0.41±0.03	0.36±0.03	0.27±0.02

RT-qPCR analysis of C/EBPβ and PPARγ. mRNA expression levels in pectoralis muscle of the experimental chicks days 6 and 8 of age (n = 7). Data with different letters indicates significant difference between groups ($P < 0.05$).